Progress Report FY2006

Core Name: Applied Marine Genomics

Project Title: Marine Organisms as Disease Vectors (MODV)

Reporting Period: 1 Oct. 2005 to 30 Sept. 2006.

Principal Investigator(s): Karen G. Burnett and Louis E. Burnett, College of Charleston

Associate Investigator(s): Bob Chapman, South Carolina Department of Natural Resources

Background and Rationale:

A major route for human exposure to marine pathogens is the consumption of fish and shellfish. The American oyster, *Crassostrea virginica*, is a vector for human pathogens, including Norwalk-like viruses, *Cryptosporidium* spp. and *Vibrio* spp. Like other filter-feeding bivalves, oysters harbor and concentrate pathogens that occur in their marine habitat and transfer them to humans when the oysters are eaten. The hazards posed by bioaccumulation of pathogens in shellfish are compounded by the traditional consumption of certain shellfish in raw or only minimally cooked dishes. There is clearly a need to understand the role of marine organisms as vectors of human disease pathogens and to develop predictive models that will more effectively assess the risk of consumption as well as target surveillance efforts to protect consumers from disease. The oyster lives in coastal waters that have highly variable water quality (e.g., low dissolved oxygen, low pH) and substantial pollutant loadings (e.g., metal and organic pollutants). Laboratory findings suggest that water quality factors such as pH and oxygen, as well as concentration of metal and organic pollutants, can impact the ability of oysters to accumulate, or biointensify, human pathogens, either externally in the mantle cavity, or internally, within the tissues of the animal.

This project will assess whether tools developed using genomics/microarray technology can be used to monitor changes in the physiological and immunological status of the oyster when influenced by environmental stressors. Furthermore, the work will document whether these physiological and immunological changes alter the abundance of opportunistic bacterial pathogens associated with the oyster. Environmental stressors to be examined include ranges of pH and O_2 conditions characteristic of estuarine environments, as well as the presence or absence of representative metal and PAH contaminants. Opportunistic *Vibrio* spp. are targeted in these studies because: (1) members of this bacterial genus, including *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus,* lead the list of bacterial pathogens responsible for outbreaks of human disease associated with shellfish consumption; (2) members of the genus are ubiquitous in aquatic and terrestrial environments; and (3) these bacteria are commonly associated with disease in a wide variety of marine organisms.

Objectives:

- Assess whether tools developed using genomics/microarray technology can be used to predict stress-induced physiological and immunological changes in oysters as a model marine organism.

- Document whether these physiological and immunological changes alter the abundance and pathogenicity of opportunistic bacterial pathogens associated with marine organisms and the ecosystem.

Accomplishments:

- In collaboration with other members of the OHH Marine Genomics Core and the Environmental Monitoring and Assessment group, research assistant Kolo Rathburn assisted in tidal creek field assessment and collected 25 oysters from each OHH sampling site. Gill and hepatopancreas from each animal at each site was preserved for transcript profiling, to be conducted in 2006-2007.
- In 2004-2005 a prototype assay was developed to monitor the distribution and fate of live bacterial pathogens injected in *C. virginica.* Oysters are injected with a known amount (usually 10^5) colony-forming units (CFU) of a strain of *Vibrio campbellii* that has been transfected with a multi-copy plasmid encoding resistance to the antibiotics chloramphenicol and kanamycin. At specified time points after injection of bacteria, the oyster is opened, its tissues homogenized and a measured amount of the homogenate is layered onto microbial culture plates containing drug-selective media. Bacterial colonies that grow on the culture plate after 24 hours are counted as CFU. A change in CFU per gram oyster tissue indicates a change in the ability of the oyster to render bacteria non-culturable, also called the bacteriostatic or killing activity. Those bacteria that cannot be detected by the CFU assay may have been removed from tissues completely (cleared); alternatively those bacteria may be still be present in tissues but unable to grow out on culture media. In order to distinguish these two possibilities, we conduct a second assay on the tissue homogenate of bacteria-injected oysters to quantify the number of bacteria that are still present in tissues, but have not been substantially degraded. This assay for "intact" bacteria is based on real-time PCR quantification of the plasmid that can be retained only by bacteria that have not been substantially degraded by the oyster's immune system. A change in the number of intact bacteria reflects a change in the ability of oysters to degrade bacteria. Thus, the pool of intact bacteria includes both culturable bacteria CFU as well non-culturable bacteria that are sufficiently intact to retain the plasmid. This prototype assay incorporates measurements of two functional components of the oyster's immune system: (1) mechanisms that render bacteria non-culturable and (2) mechanisms that degrade bacteria. It is important to distinguish between these two mechanisms, since it is possible that, under favorable conditions, non-culturable bacteria may become culturable pathogens again. In addition, non-culturable bacteria may still have pathogen effects on a host species.
- In 2005-2006 studies using the bacterial challenge assay, as detailed above, provided a striking picture of seasonal and environmental impacts on the ability of oysters to

eliminate bacterial pathogens. We currently assess safety of oysters for consumption by CFU or other quantification of targeted bacteria (such as enterococci and fecal coliforms). The data we present below suggests that the abundance and persistence of a pathogen within an oyster varies in a predictable pattern with environmental and seasonal conditions. End-users of these data include the OHH Pathogen Source Tracking Project, as well as public health groups such as DHEC.

Hypoxia as a natural stressor (laboratory study). Postdoctoral research associate Brett Macey showed that oysters held at sub-lethal levels of hypoxia (20% of air saturation) with high levels of CO_2 and low pH (hypercapnic hypoxia, HH) retained higher levels of culturable bacteria in their tissues than did animals maintained in air-saturated water over 60 min after injection of bacteria (**Figure 1**; 2-way ANOVA, p=0.003; *significant pairwise differences between treatments, Holm-Sidak method).

- High numbers of intact bacteria could still be detected by real-time PCR in both normoxia- and HH-exposed animals throughout the 60 min timecourse. By 60 min after injection, the percentage of intact bacteria that remained culturable (CFU/intact X 100%) was significantly higher in oysters exposed to HH (**Figure 2**). Taken together these data suggested that exposure to HH impairs mechanisms of bacteriostasis in oyster tissues. This laboratory experiment also demonstrated that the bacterial clearance assay can be used quantify the increased burden of bacterial pathogens in oysters as a function of a natural stressor, such as environmental hypoxia accompanied by naturally co-occurring hypercapnic hypoxia and acidosis. These data will be important in developing quantitative models of human exposure to pathogens in hypoxic environments.

- Metal contaminant effects (laboratory). Master's degree candidate Heidi Williams exposed oysters to an environmentally-relevant dose of cadmium (50 ppb) at 25^{o}C in 25ppt seawater for 30 days, then used the bacterial clearance assay to examine bacteriostatic activity of cadmium-exposed as compared to unexposed oysters. There was a significant effect of time and but no significant effect of cadmium-exposure (two way ANOVA), on the ability of oysters to render bacteria non-culturable (**Figure 3**), although there was a significantly higher number of culturable bacteria in the tissues of cadmium-exposed animals at 30 min after injection, compared to control, unexposed animals. Interestingly, cadmium-exposed animals did have significantly lower total hemocyte counts mL^{-1} hemolymph than did animals held for 30 days without cadmium exposure (**Figure 4**; one-way ANOVA; p = 0.038).

- Seasonal effects (field assessment). Brett Macey, Heidi Williams, and Research Assistant Kolo Rathburn recently completed a year-long study to examine seasonal differences in the ability of oysters to render bacteria non-culturable and to degrade these injected bacteria. Oysters were collected from a relatively pristine site, Schooners Creek, SC, every 6 – 8 weeks from November 2005 to late September 2006. After three days in the laboratory, animals were injected with *V. campbellii* and CFU mL^{-1} tissue quantified at 60 min after injection of bacteria. Oysters displayed significant differences in bacterial clearance as a function of season and/or water temperature (**Figure 5**; one-way ANOVA, p<0.001; Holm-Sidak method).

Animals collected in January 2006 were much less able to inactivate bacteria than animals collected in other seasons from the same field site.

- Water quality effects (field assessment). Oysters collected from OHH tidal creek sites by the monitoring and assessment group were tested in the bacterial clearance assays by Brett Macey, Heidi Williams, research assistant Kolo Rathburn and summer student Thomas Miller. Oysters collected from Guerin Creek, a forested reference site, retained significantly lower numbers of culturable bacteria at 60 min after injection of bacteria than oysters from any other creek (**Figure 6**; one-way ANOVA, $p<0.001$). The density of hemocytes in hemolymph among oysters from the various tidal creeks did not differ significantly (**Figure 7**; one-way ANOVA, $p=0.991$). Notably, average total hemocyte count (THC) mL^{-1} hemolymph of oysters from tested tidal creeks was negatively correlated with the average number of culturable *Vibrio campbellii* g^{-1} tissue recovered at 60 min after injection of 10^5 bacteria per animal. The observed negative correlation (**Figure 8**; linear regression, $r^2 = -0.831$) is statistically significant ($p<0.001$). There was a strong positive correlation ($r=0.740$; $p=0.02$) between Enterococci contamination of whole oyster tissues (but not water concentrations of Enterococci) and average CFU *V. campbellii* g−1 tissue recovered at 60 min after injection of bacteria (**Figure 9**). In other words, high pathogen burden in oyster tissues correlated with reduced ability the oyster to eliminate pathogens.

- Neither THC mL^{-1} hemolymph nor CFU g^{-1} oyster tissue were significantly correlated with pathogen loads in the surrounding water (Pathogen Source Tracking Project). Correlations with other environmental assessments, including water and oyster tissue pathogens will be assessed as data become available.

- Distribution of invasive bacterial pathogens in oyster tissues. In 2004-2005 CofC graduate student Heidi Williams developed a prototype assay to monitor tissue distribution and fate of live bacterial pathogens in *C. virginia.* In 2005-2006, she used this assay used to show that oyster tissues play distinct roles in the elimination of invading pathogens. While injected bacteria are distributed to and rapidly eliminated from all tissues of the oyster (data not shown), the mantle retains a larger percentage of the total culturable bacteria that can be recovered from all tissues at 60 and 120 min after injection (**Figure 9**). The results of these experiments will be critical to understanding the processes that alter the persistence of bacterial pathogens in oysters and other marine species harvested for human consumption. End-users of these data include the OHH Pathogen Source Tracking Project, as well as public health groups such as DHEC.

Publications/Presentations:

1. Macey, B.M., Burnett, L.E., Burnett, K.G. 2006. Effects of hypercapnic hypoxia on the clearance of *Vibrio campbellii* in the Eastern oyster, *Crassostrea virginica.* 31st Annual Eastern Fish Health Workshop. Charleston, SC. March 2006.

2. Macey, B.M., Burnett, L.E. Burnett, K.G. 2006. Effects of hypercapnic hypoxia on the clearance of *Vibrio campbellii* in the Eastern oyster, *Crassostrea virginica.* Tenth Congress of the International Society of Developmental and Comparative Immunology. Charleston, SC. July 2006.

Meeting Abstracts Submitted:

1. Macey, B.M., Miller, T.E., Williams, H., Rathburn, C.K., Burnett, L.E. and Burnett, K.G. 2006. Influence of water quality on immune function in the Eastern oyster, *Crassostrea virginica.* International Conference on Shellfish Restoration. Charleston, SC. November 2006.

2. Macey, B.M., Miller, T.E., Williams, H., Rathburn, C.K., Burnett, L.E. and Burnett, K.G. 2006. Influence of water quality on immune function in the Eastern oyster, *Crassostrea virginica.* Annual Society of Integrative and Comparative Biology Meeting. Phoenix, AZ. January 2007.

3. Williams, H.R., Macey, B.M., Rathburn, C.K., Burnett, L.E. and Burnett, K.G. 2007. The fate of *Vibrio campbellii* within the Eastern oyster *Crassostrea virginica* (Gmelin). 99th Annual National Shellfisheries Association Meeting, February 2007.

Application/Technology Transfer Relevant to OHH Strategic Goals:

1.0 Scientific Research and Application

The data generated from these studies will be important in developing quantitative models of human exposure to pathogens in the marine environment. The results will provide critical understanding as to whether and how environmental metals and hypoxia can alter the ability of oysters to eliminate or inactivate bacterial pathogens in their own tissues and in the ecosystem. End-users of these data include the OHH Pathogen Source Tracking Project, as well as public health groups such as state health officials. Furthermore, data from these studies will be used to develop and test new microarray-based tools for assessing ecosystem health.

Project Abstract:

In this project researchers are testing whether changes in the coastal marine environment, such as low oxygen (hypoxia), metals and organic pollutants can increase the risk that oysters, or other marine fish and shellfish, will pass disease-causing microorganisms (pathogens) to humans. Like other filter-feeding bivalves such as clams and mollusks, oysters harbor and concentrate pathogens that occur in their marine habitat. Through direct contact with the oysters and their surrounding water or consumption of oysters humans can be exposed to these pathogens. Previous studies conducted in several laboratories have shown that poor water quality, such as low oxygen levels, low pH, metal and organic pollutants, can impair some of the specific mechanisms that oysters use to kill or inactivate these bacterial pathogens.

At the end of Year Two, MODV personnel have developed and implemented a method for quantifying the ability of oysters to eliminate bacteria that penetrate their tissues. Studies using this method suggest that exposure to low oxygen and less so to cadmium suppresses the ability of oysters to eliminate culturable bacteria from their tissues. This assay has been translated into a field health assessment, showing that the ability of oysters to eliminate invading bacteria varies as a function of season and of tidal creek source. Furthermore, preliminary data suggest that when the ability of an oyster to eliminate bacteria is compromised by environmental or seasonal stressors, oyster tissue burdens of human disease pathogens, such as Enterococci, increases. These data will contribute to the development of a mathematical model that predicts the increased risk of exposure to pathogens when humans consume or come in contact with oysters and the coastal waters they inhabit. These data have been presented at one regional and one national meeting, and abstracts have been submitted for three additional national/international meetings in early 2007.

Unresolved Issues: None.

Budget Report (9/30/06):

	Total Budgeted Years 1-2	**Actual Year 1 Expenses**	**Actual Year 2 Expenses**	**Total Spent through Year 2**		**Year 3 Budgeted**
Total	413,191	88,685	172,101	260,786		$212,337
Salaries & Fringe Benefits	272,971	68,576	124,799	193,375		53,698
Travel	10,800	1,183	1,890	3,073		0
Supplies	68,000	1,496	19,332	20,828		5,000
Indirect Costs	61,420	15,430	28,079	43,509		12,082
Subcontract (Terwilliger)	-	-	-	-		58,357
Contract (Adams)	-	-				82,915

Hypoxia as a natural stressor on clearance of culturable bacteria and degradation of bacterial pathogens (laboratory study) – Figures 1, 2

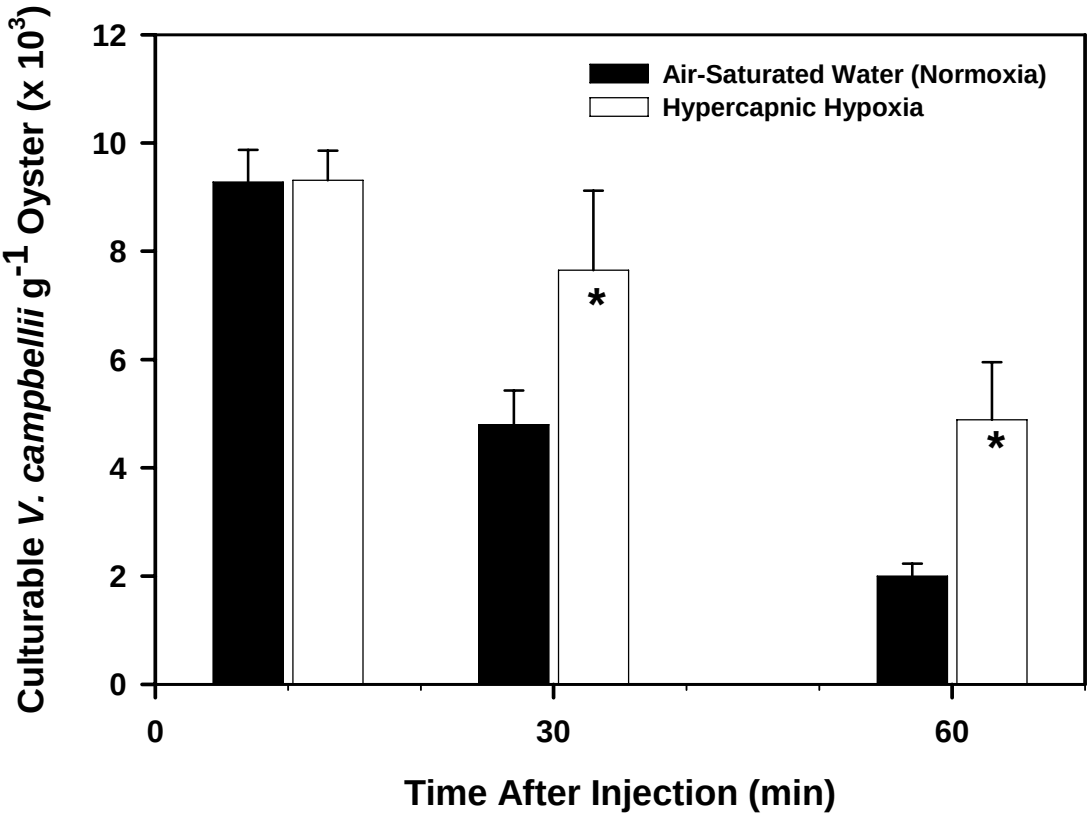

Figure 1. Mean number of culturable *Vibrio campbellii* g^{-1} whole oyster tissue (± SEM) recovered from oysters held in air-saturated versus hypercapnic hypoxic waters. Oysters were exposed to air-saturated water (pH 7.8-8.0) or to hypercapnic hypoxia (HH; 20% of air saturation, pH 6.2 – 6.5) at 25 °C for 4 hours, then injected with 10^5 live *V. campbellii.* HH-exposed oysters retained higher levels of culturable bacteria in their tissues than did animals maintained in air-saturated water over 60 min after injection of bacteria (**Figure 1**; 2-way ANOVA, p=0.003; *significant pairwise differences between treatments, Holm-Sidak method).

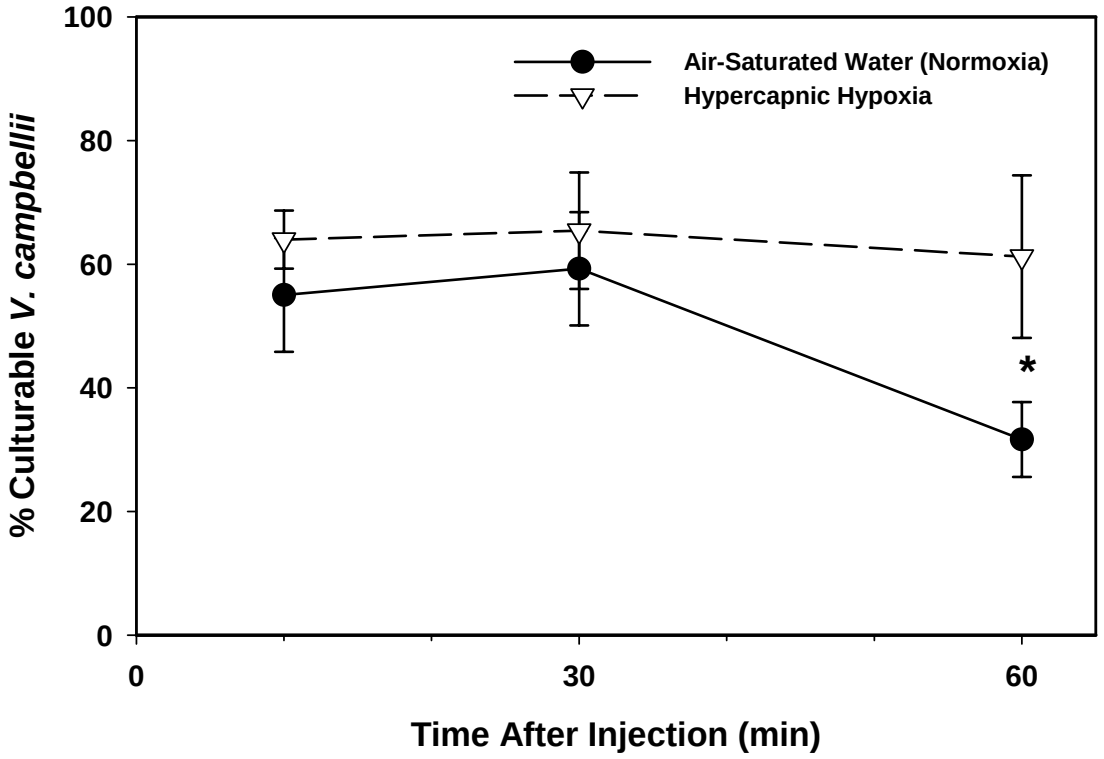

Figure 2. Mean percentage of intact *Vibrio campbellii* using real-time PCR (± SEM) in whole oyster tissues that were culturable using selective plating at 10, 30 and 60 min after injection of bacteria. Oysters were exposed to air-saturated water (pH 7.8-8.0) or to hypercapnic hypoxia (HH; 20% of air saturation, pH 6.2 – 6.5) at 25 °C for 4 hours, then injected with 10^5 live *V. campbellii.* Oysters held in HH retained a significantly higher percentage of culturable *V. campbellii* than animals held in air-saturated water at 60 min (2-way ANOVA, p=0.027; *significant pairwise differences between treatments, Holm-Sidak method).

Summary (Figures 1 and 2):

These data clearly demonstrate that bacterial pathogens persist in higher numbers in oysters exposed to hypercapnic hypoxia. Furthermore, HH reduces the ability of an oyster to render pathogenic bacteria non-culturable.

Effects of cadmium exposure on clearance of culturable bacteria and total hemocyte counts mL^{-1} hemolymph (laboratory study) – Figures 3, 4

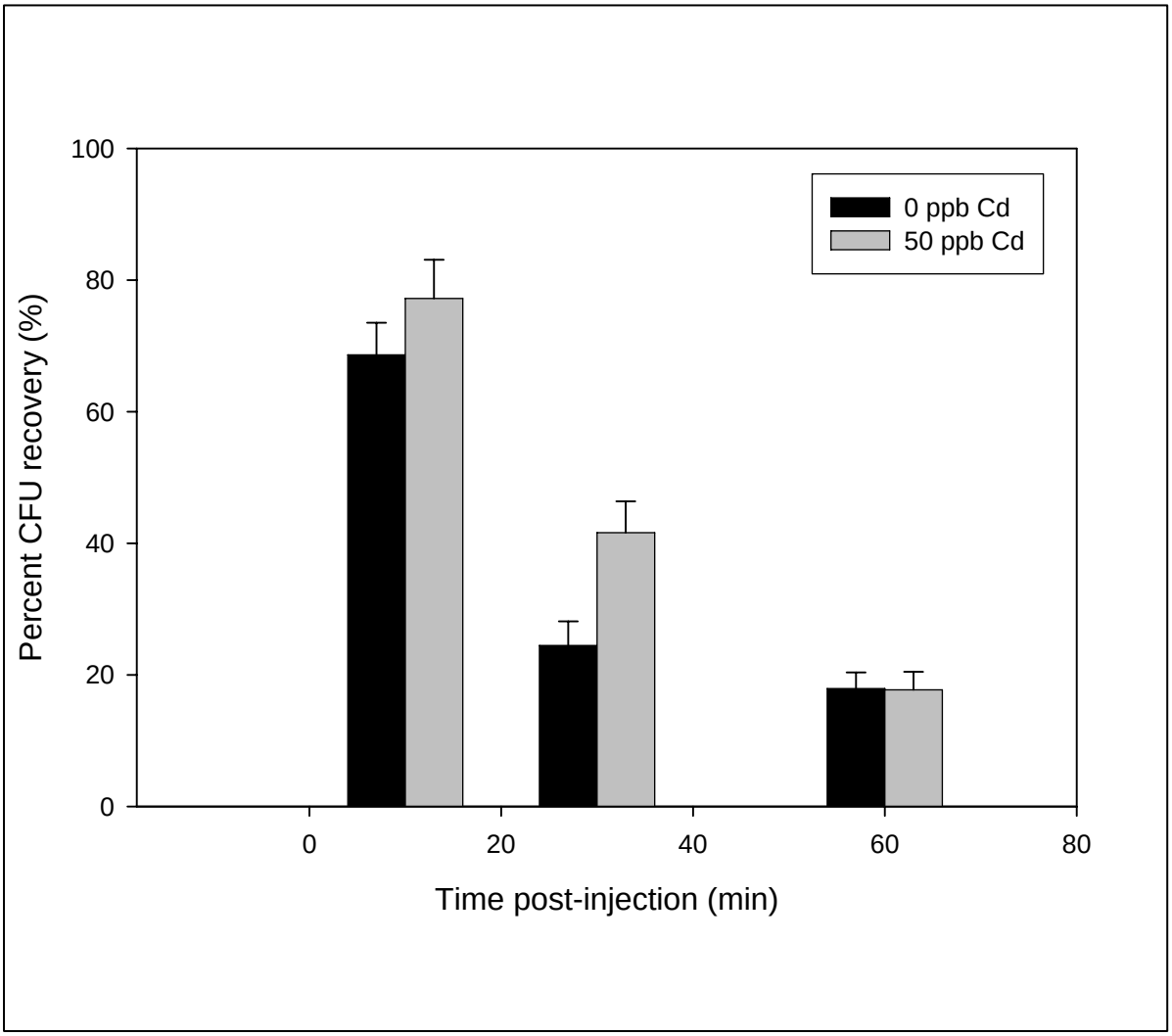

Figure 3. Mean percentage of intact *Vibrio campbellii* (± SEM) recovered from the tissues of whole oysters exposed to 50 ppb or 0 ppb cadmium for 28 days prior to bacterial challenge. There was a significant effect of time and but no significant effect of cadmium-exposure (two-way ANOVA, $p = 0.08$), on the ability of oysters to render bacteria non-culturable, although there was a significantly higher number of culturable bacteria in the tissues of cadmium-exposed animals at 30 min after injection (one-way ANOVA, $p = 0.016$) compared to control, unexposed animals.

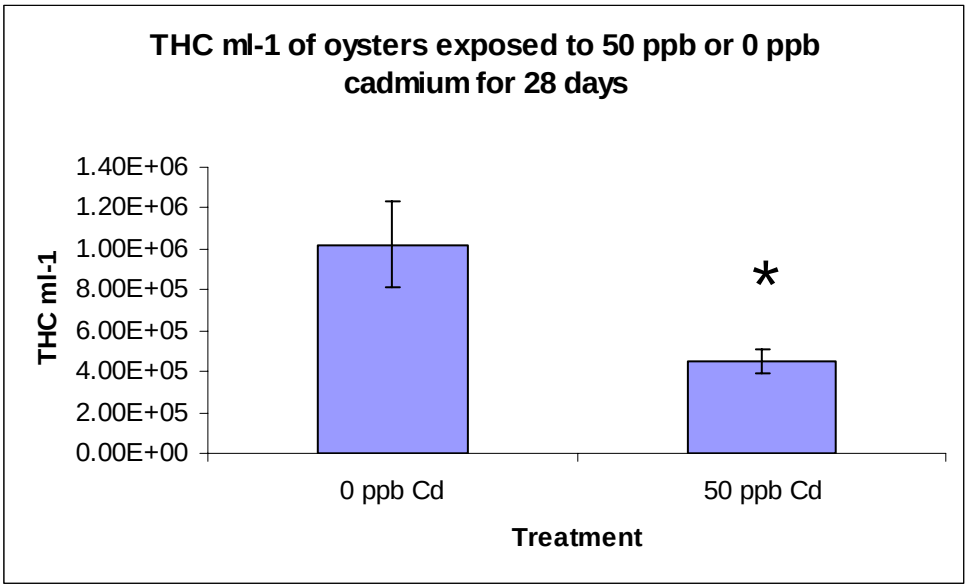

Figure 4. Mean total hemocyte count (THC) mL^{-1} hemolymph (± SEM) in oysters exposed for 28 days to 50 or 0 ppb cadmium. Exposure to cadmium significantly reduced total hemocyte count in cadmium-exposed animals (one-way ANOVA, $p = 0.036$).

Seasonal effects on clearance of culturable bacteria (field assessment) – Figure 5

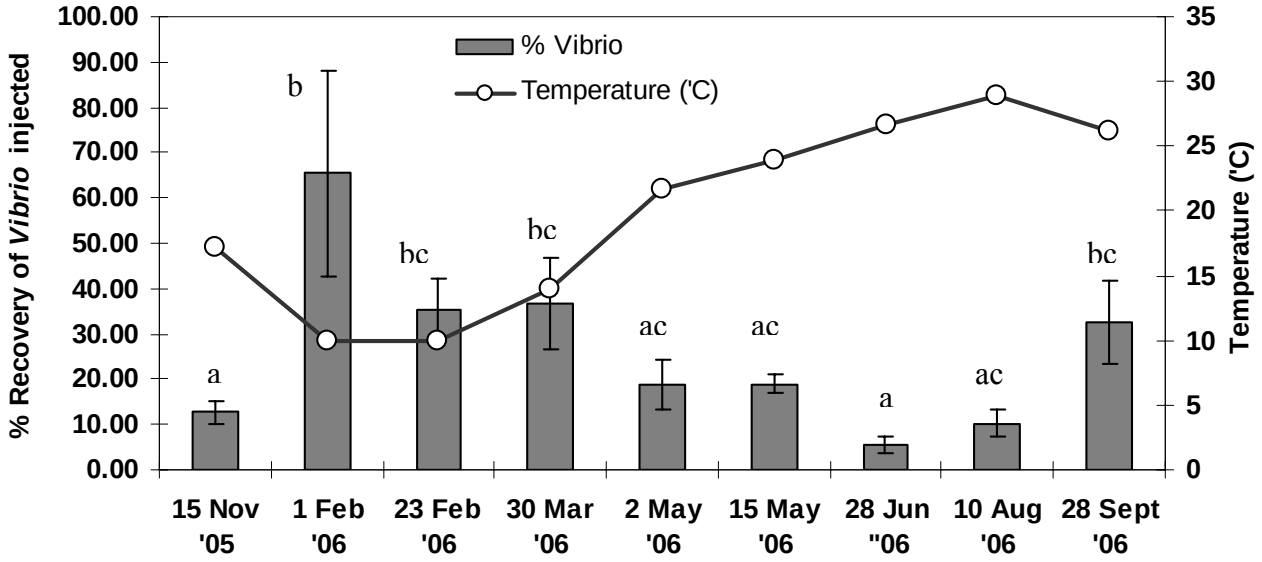

Figure 5. Mean percentage recovery (± SEM) of culturable *Vibrio campbellii* from oysters 60 min after injection of 10^5 bacteria into the adductor muscle as a function of water temperature/season. Oysters were collected from Schooners Creek from November 2005 to September 2006. Each bar represents the mean +/- SEM percent recovery of the injected dose for 6 – 8 oysters. Oysters displayed significant differences in bacterial clearance as a function of season and/or water temperature (one-way ANOVA, $p<0.001$; different letters indicate a significant difference between values, Holm-Sidak method).

Water quality effects on clearance of culturable bacteria and total hemocyte counts mL $^{-1}$ hemolymph (field assessment) – Figures 6, 7, 8, 9.

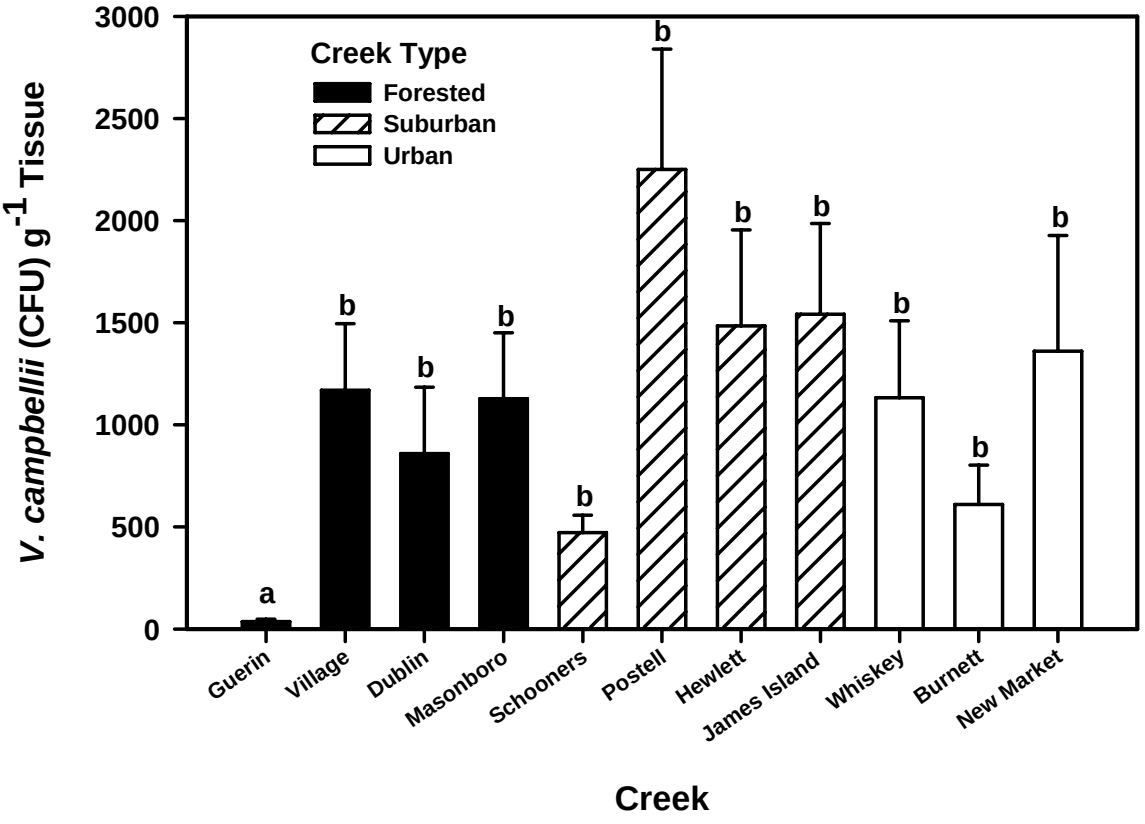

Figure 6. Mean number of culturable *Vibrio campbellii* g^{-1} whole oyster tissue (± SEM; n=6-8 oysters from each tidal creek) recovered from oysters 60 min after injection of 10^5 bacteria into the adductor muscle. Oysters were collected in June and July, 2006, from sites in South Carolina and North Carolina that were classified as either "forested," "suburban" or urban, by the Environmental Monitoring and Assessment Group (DiDonato *et al*., pers. comm.). Oysters collected from Guerin Creek retained significantly lower numbers of culturable bacteria at 60 min after injection of bacteria than oysters from any other creek (one-way ANOVA, $p<0.001$; different letters indicate a significant difference between values, Holm-Sidak method).

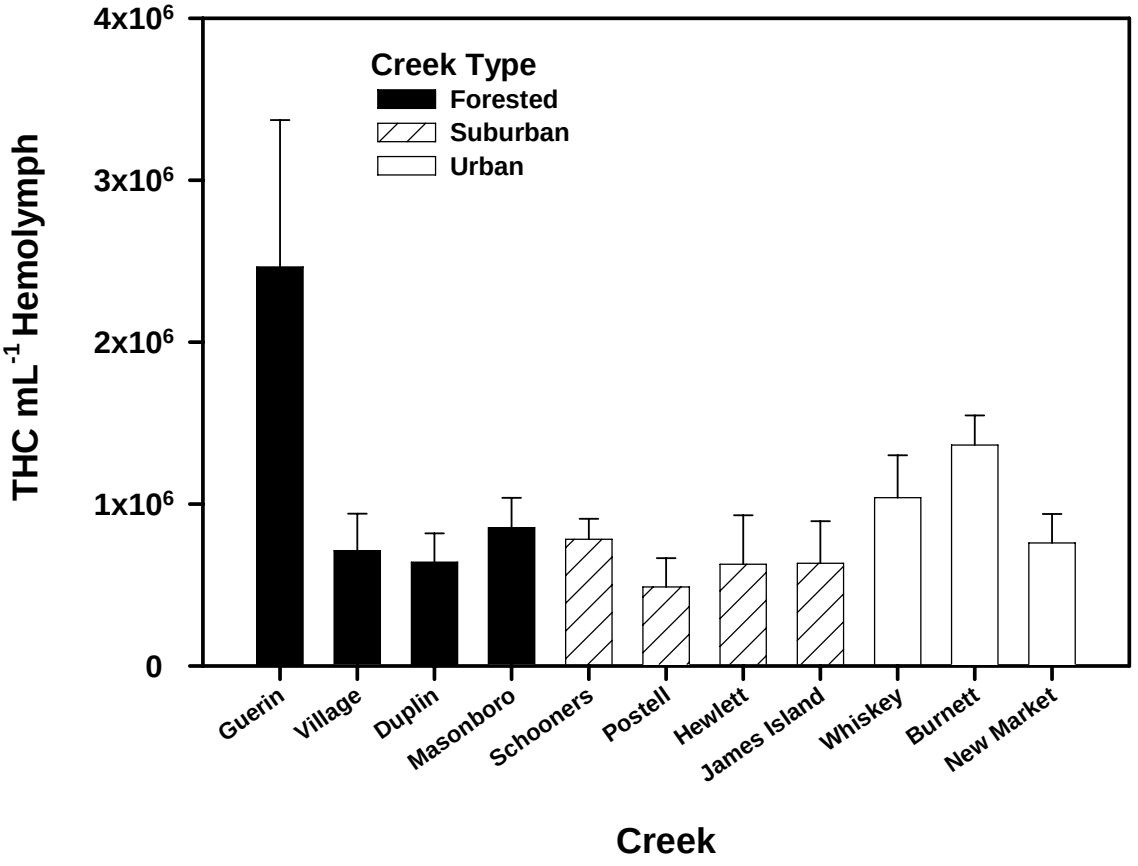

Figure 7. Total hemocyte count (THC) mL^{-1} hemolymph of oysters among the different tidal creeks. Each bar represents the mean ± SEM THC mL^{-1} hemolymph of 6-8 oysters from each tidal creek. Oysters were collected in June and July, 2006, from sites in South Carolina and North Carolina that were classified as either "forested," "suburban" or urban, by the Environmental Monitoring and Assessment Group (DiDonato *et al*., pers. comm.). The density of hemocytes in hemolymph among oysters from the various tidal creeks did not differ significantly (one-way ANOVA, p=0.991).

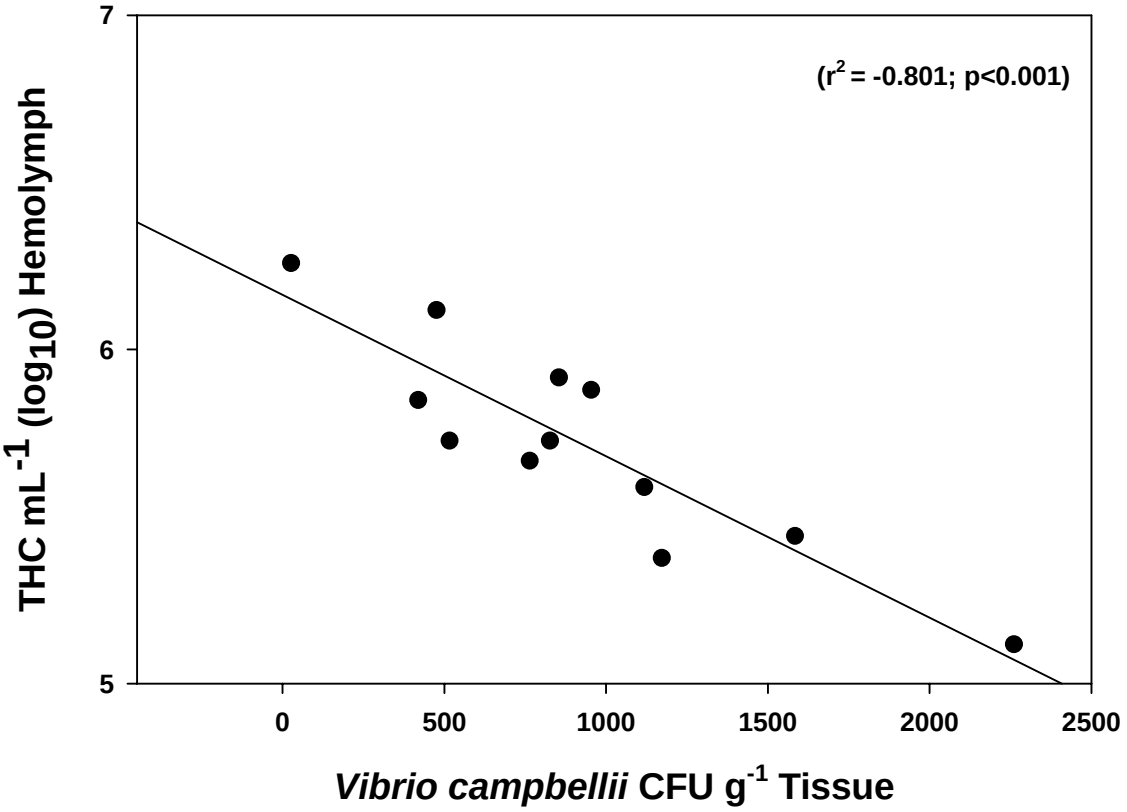

Figure 8. The correlation of average total hemocyte count (THC) mL^{-1} hemolymph of oysters from tested tidal creeks with average number of culturable *Vibrio campbellii* g^{-1} tissue recovered at 60 min after injection of 10^5 bacteria per animal. The observed negative correlation (linear regression, $r^2 = -0.831$) is statistically significant ($p<0.001$).

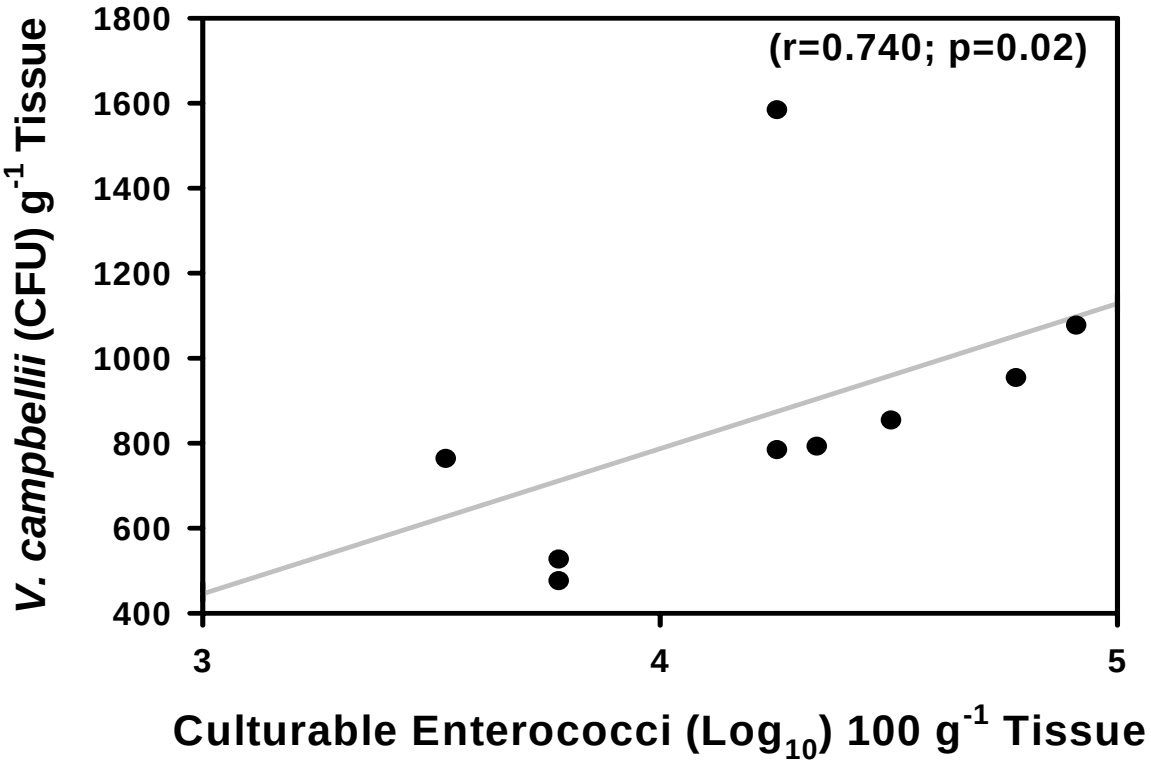

Figure 9. The correlation of culturable Enterococci 100 g^{-1} oyster tissue with average CFU *V. campbelli*i g^{-1} tissue recovered at 60 min after injection of bacteria. There was a strong positive correlation (r=0.740; p=0.02) between Enterococci contamination of whole oyster tissues and the antibacterial activity of oysters

Distribution and elimination of invasive bacterial pathogens in oyster tissues – Figure 10.

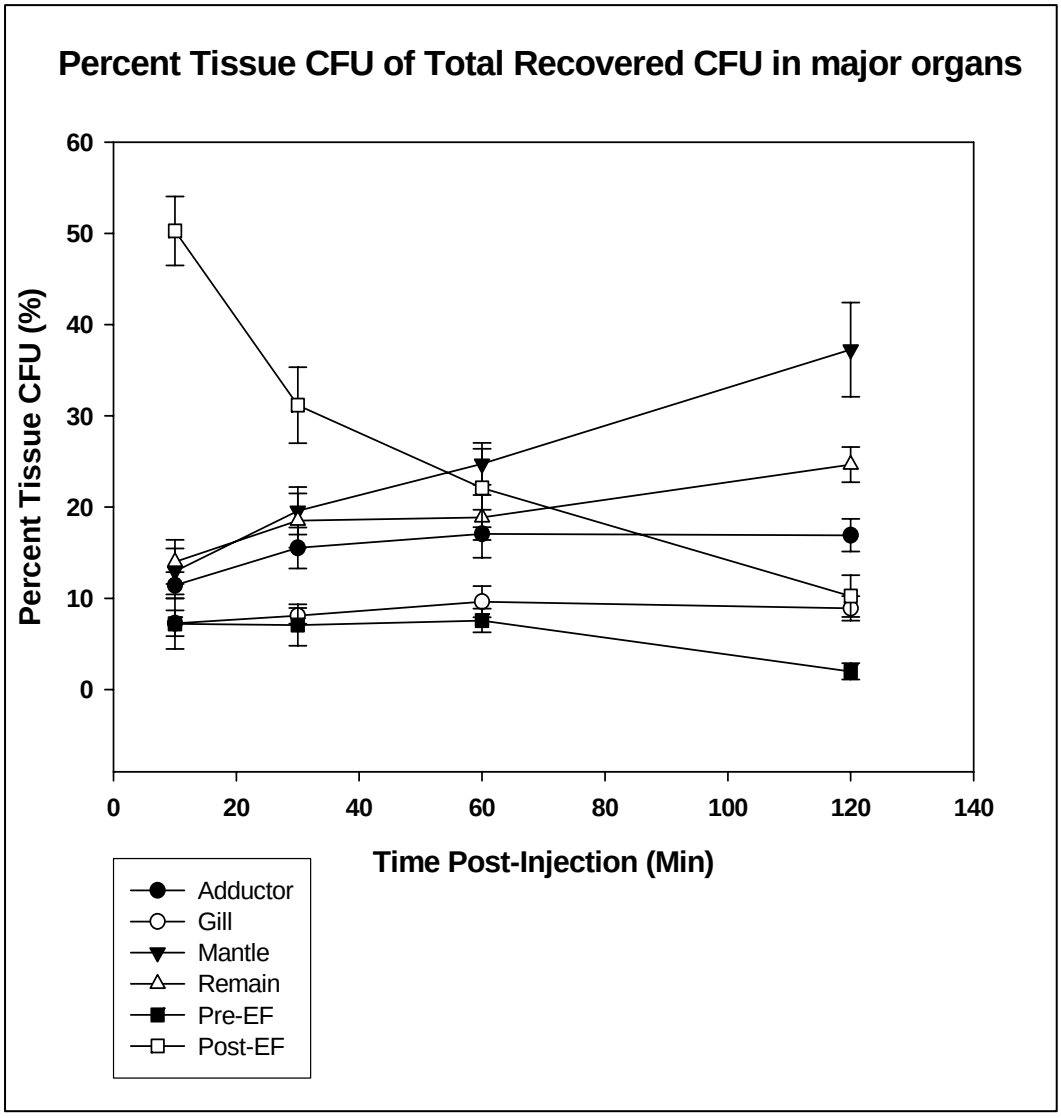

Figure 10. Distribution of recovered culturable bacteria in oysters, expressed as the mean percent of CFU g^{-1} tissue ± SEM. The adductor muscle, which is the site of injection, rapidly loses its high initial density of culturable bacteria and most tissues retain a consistent number of CFU g^{-1} tissue through the 120 min timecourse. In contrast, an increasingly higher percentage of the total culturable bacteria is recovered from the mantle over the 120 min following bacterial challenge. Pre-EF = extracellular fluid prior to severing the adductor muscle; post-EF = extracellular fluid after major tissues have been dissected; remain = other tissues, including hepatopancreas, labial palps, heart, gonads.